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Genetic Analysis Reveals That Both Haemagglutinin and Neuraminidase Determine the Sensitivity of Naturally Occurring Avian Influenza Viruses to Zanamivir *in Vitro*

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The basis of differential sensitivity of replication of influenza viruses to the neuraminidase-specific inhibitor zanamivir was examined using four avian influenza viruses and reassortants produced between them. IC_{50} values for inhibition of neuraminidase activity by zanamivir were similar for each of the four viruses, whereas the haemagglutinating activity of each of the viruses was relatively insensitive to zanamivir. However, the four viruses showed distinct zanamivir-sensitivity profiles in tissue culture. Analysis of the reassortant viruses showed that sensitivity was determined by the haemagglutinin gene (segment 4) and the neuraminidase gene (segment 6) and was independent of the remaining six RNA segments. Decreased sensitivity to zanamivir was associated with possession of a haemagglutinin that is released from cells with decreased dependence on neuraminidase and with possession of a neuraminidase that has a short stalk region.

INTRODUCTION

Influenza A viruses cause respiratory disease in humans and infect other mammals and birds. They are members of the orthomyxoviridae, possessing a negative-sense, single-stranded RNA genome of eight segments. Segments 4 and 6 encode the viral envelope glycoproteins haemagglutinin (HA) and neuraminidase (NA), respectively. HA binds to sialic acid-bearing receptors on the host cell surface through a broad, shallow surface pocket of conserved amino acids near the membrane distal tip of each subunit (Weis *et al.,* 1988). The existence of a secondary sialic acid binding site has also been postulated (Sauter *et al.,* 1992). NA consists of a cytoplasmic tail, a hydrophobic transmembrane span, external stalk, and globular head. The enzymatic site in the head catalyses the removal of terminal sialic acid residues from an α-2,3 or α-2,6 ketosidic linkage to an adjacent sugar residue on a glycoconjugate, destroying virus–receptor and virus–virus interactions, and thus facilitating the release of infectious, nonaggregated progeny virus. Influenza A viruses are classified on the basis of their surface antigens, HA and NA, there being 15 HA subtypes (H1–H15) and 9 NA subtypes (N1–N9). Viruses that have become established in mammals show a restricted combination of HA and NA subtypes, but all HA and NA subtypes are represented among avian influenza viruses. Annual epidemics of human influenza are associated with amino acid substitutions in the antigenic sites leading to an accumulation of changes and antigenic drift. Rarely, but dramatically, reassortment between influenza viruses results in the exchange of genes, so HA and/or NA can be introduced into a new genetic background, resulting in antigenic shift. Recently, a virus of avian origin (A/HongKong/156/97; H5N1) was responsible for the death of a small number of people in Hong Kong (Subbarao *et al.,* 1998; Claas *et al.,* 1998), exemplifying the potential of avian viruses to cross to the human population. The development of prophylactic agents to protect against infection by all strains of influenza A and B viruses, and to treat such infections, thus is of prime importance.

Sialic acid-based compounds have previously been shown to restrict the replication of influenza viruses in tissue culture (Meindl *et al.,* 1971; Palese and Compans, 1976). Further modifications of the sialic acid molecule led to the development of high-affinity, high-specificity inhibitors of the catalytic activity of influenza virus NA (von Itzstein *et al.,* 1993). 4-Guanidino-2,4-dideoxy-2,3-dehydro-*N*-acetylneuraminic acid (zanamivir) is an unsaturated analog of sialic acid, with a guanidino group substituting for the hydroxyl group at position C4 on sialic acid. It binds reversibly to the enzyme with an inhibition constant (K_i) of 2×10^{-10} M (von Itzstein *et al.,* 1993), and the interactions between this compound and the NA active site have been characterised by X-ray crystallography. The conformation of binding is somewhat different from that of sialic acid, and the arrangement of functional groups mimics more those of the transition state than those of the ground state conformation of sialic acid.

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Zanamivir has been shown to be effective *in vivo* in mice and ferrets, decreasing pyrexia and virus shedding after infection with human influenza A or B viruses (Woods *et al.,* 1993; Ryan *et al.,* 1995), and in mice after infection with A/HongKong/156/97 (Gubareva *et al.,* 1998). In chickens, intranasal administration of zanamivir and virus showed some protective effect (Gubareva *et al.,* 1995), but intratracheal administration of zanamivir and virus gave no protection against highly pathogenic avian viruses (McCauley *et al.,* 1995). These observations reflect the fact that although zanamivir, a locally acting drug, may be effective in treating respiratory tract infections induced by many influenza viruses, it is much less effective in treating the systemic infection caused by highly pathogenic avian influenza viruses.

Zanamivir-resistant variants have been selected after repeated passage in the presence of the drug in tissue culture. Resistant viruses with an NA that is less sensitive to inhibition by zanamivir have been isolated (Staschke *et al.,* 1995; Blick *et al.,* 1995; Gubareva *et al.,* 1996). Additionally, resistant viruses with decreased dependence on NA for release from the cell surface have been produced (McKimm-Breschkinn *et al.,* 1996; Gubareva *et al.,* 1996).

Naturally occurring influenza viruses show variation in sensitivity of their replication to zanamivir in tissue culture. We previously analysed the sensitivity of avian influenza viruses with NA glycoproteins from each of the nine NA subtypes (Thomas *et al.,* 1994). In three assays of sensitivity of virus replication to zanamivir, each of the viruses showed sensitivity to the drug, but the extent of sensitivity differed depending on both the virus and the assay, so each virus had a distinct sensitivity profile. Because the enzyme activities of avian influenza virus NAs of all subtypes are all similarly sensitive to inhibition by zanamivir (Gubareva *et al.,* 1995), the differences in sensitivity in tissue culture would indicate that avian influenza viruses differ in their dependency on NA for replication *in vitro*.

In view of the differential sensitivity of naturally occurring avian influenza viruses to zanamivir, and their likely importance in future pandemic influenza in humans, the aim of this study was to identify the genes that contribute to the variation in sensitivity of naturally occurring avian influenza viruses to zanamivir *in vitro* and to define the mechanism by which these genes influence sensitivity.

RESULTS AND DISCUSSION

To identify the genes that contribute to the variation in sensitivity of influenza viruses to zanamivir *in vitro*, we initially determined the sensitivity profiles of four naturally occurring avian influenza viruses in five *in vitro* assays.

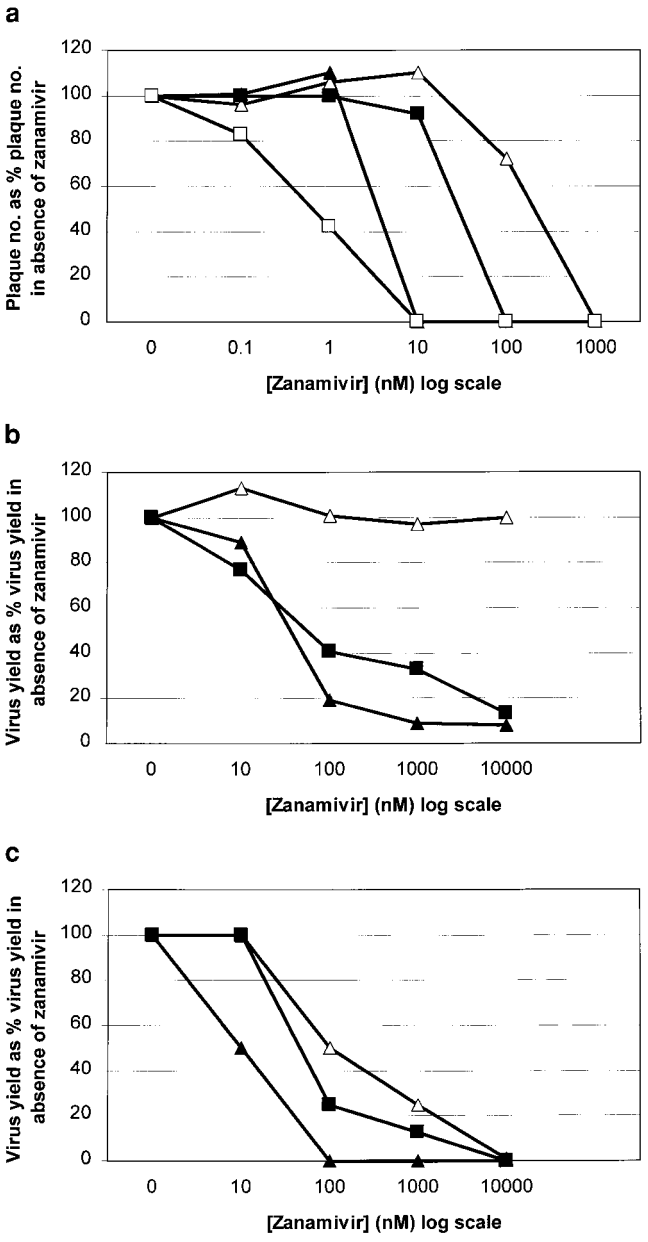


FIG. 1. Effect of zanamivir on replication and spread in MDCK cells of avian influenza viruses in assays of plaque number (a), virus yield measured by infectivity titer on CEF cells (b), and virus yield measured by haemagglutination titer (c). Viruses examined were SD17 (△), Langham (■), Egypt (▲), and Duck Ireland (□).

SD17 is the least sensitive virus in Madin–Darby canine kidney cells

The effect of zanamivir on virus replication in Madin–Darby canine kidney (MDCK) cells was examined in multicycle plaque formation assays (Fig. 1a) and in single-cycle virus yield assays. Virus yield was measured by either infectivity titer (Fig. 1b) or by haemagglutination titer (Fig. 1c). Mean IC_{50} values, for inhibition of replication of each virus in each assay, are given in Table 1. Each virus showed greater sensitivity in the plaque as-

TABLE 1

Sensitivity of Avian Influenza Viruses to Zanamivir

Virus	HA and NA subtype	Effect of zanamivir						
		NA activity		Haemagglutination titre	Plaque formation in MDCK cells IC_{50} (nM)	Virus release from MDCK cells measured by infectivity IC_{50} (nM)	Virus release from MDCK cells measured by haemagglutination IC_{50} (nM)	Aggregation of virus at MDCK cell surface in presence of 1 μM zanamivir
		IC_{50} (nM)	IC_{90} (nM)					
SD17	H7N1	7.9 ± 1.4	96.1 ± 4.0	No effect	147 ± 11[a]	>10 000	178 ± 0	Small aggregates
Langham	H7N3	9.2 ± 2.7	86.8 ± 4.7	No effect	28 ± 8	304 ± 53	101 ± 13	Large aggregates
Egypt	H7N1	9.0 ± 0.7	76.7 ± 4.8	No effect	3.2 ± 0	200 ± 46	8.8 ± 1.8	Large aggregates
Duck Ireland	H5N8	5.5 ± 0	48.9 ± 3.3	Decreased 2-fold with >10 nM	1.3 ± 0.3	ND	ND	ND

Note. ND, Not done.

[a] IC_{50} and IC_{90} values given as mean ± SEM.

say than in the virus yield infectivity titer assay, an effect previously observed for sensitivity of influenza virus replication to the zanamivir analog FANA in MDBK cells (Palese *et al.,* 1974). This probably reflects amplification of inhibitory effects of drug in the multiple rounds of virus replication in a plaque assay. The inhibitory effect of zanamivir on virus yield was more apparent in the haemagglutination titer assay than in the infectivity titer assay. This is probably because for titration of infectivity, the sample, and therefore zanamivir, was diluted by a factor of 10^6-10^8, so clumps of virus could disaggregate and plaque formation was not inhibited. However, for measuring haemagglutination titer, the dilution factor was 10^1-10^3, so zanamivir would have remained at a concentration sufficient to prevent the disaggregation of clumps of virus, which could not efficiently agglutinate erythrocytes.

The rank sum test was used to analyse the differences in IC_{50} values. In the plaque assay (Fig. 1a), SD17 was less sensitive than Langham ($P < 0.01$), Egypt ($P < 0.05$), and Duck Ireland ($P < 0.01$); Langham was less sensitive than Egypt ($P < 0.05$) and Duck Ireland ($P < 0.05$); and there was no significant difference in sensitivity between Egypt and Duck Ireland.

In the virus yield infectivity titer assay (Fig. 1b), SD17 was less sensitive than Langham ($P < 0.05$) and Egypt ($P < 0.01$), but Langham and Egypt showed no significant difference in sensitivity. However, in the virus yield haemagglutination titer assay (Fig. 1c), SD17 was less sensitive than Langham ($P < 0.05$) and Egypt ($P < 0.05$), and Langham was less sensitive than Egypt ($P < 0.05$). Because Duck Ireland grew to low infectivity titres *in ovo* (10^5 pfu/ml), this virus could not be used in the single cycle yield assay, which required high m.o.i.

The release of virus from infected cells was also examined by electron microscopy. In zanamivir-free medium, nonaggregated virus particles were observed budding at the surface of MDCK cells infected with SD17, Langham, or Egypt (Figs. 2a, 2c, and 2e). In cells infected with Duck Ireland, virus budding at the plasma membrane was not seen, probably due to the low m.o.i. and failure to observe the low number of virus-infected cells. In the presence of 1 μM zanamivir, SD17 virus particles formed very small aggregates at the cell surface (Fig. 2b), whereas Langham and Egypt formed large aggregates (Figs. 2d and 2f).

To summarise, SD17 was the least sensitive virus in each of three tissue culture assays, whereas Langham exhibited an intermediate sensitivity. Egypt showed an intermediate sensitivity in the virus yield infectivity titer assay but was highly sensitive in the virus yield haemagglutination titer assay and in the plaque assay, behaving similarly to Duck Ireland in the latter assay. Increased sensitivity was associated with aggregation of virus particles at the cell surface.

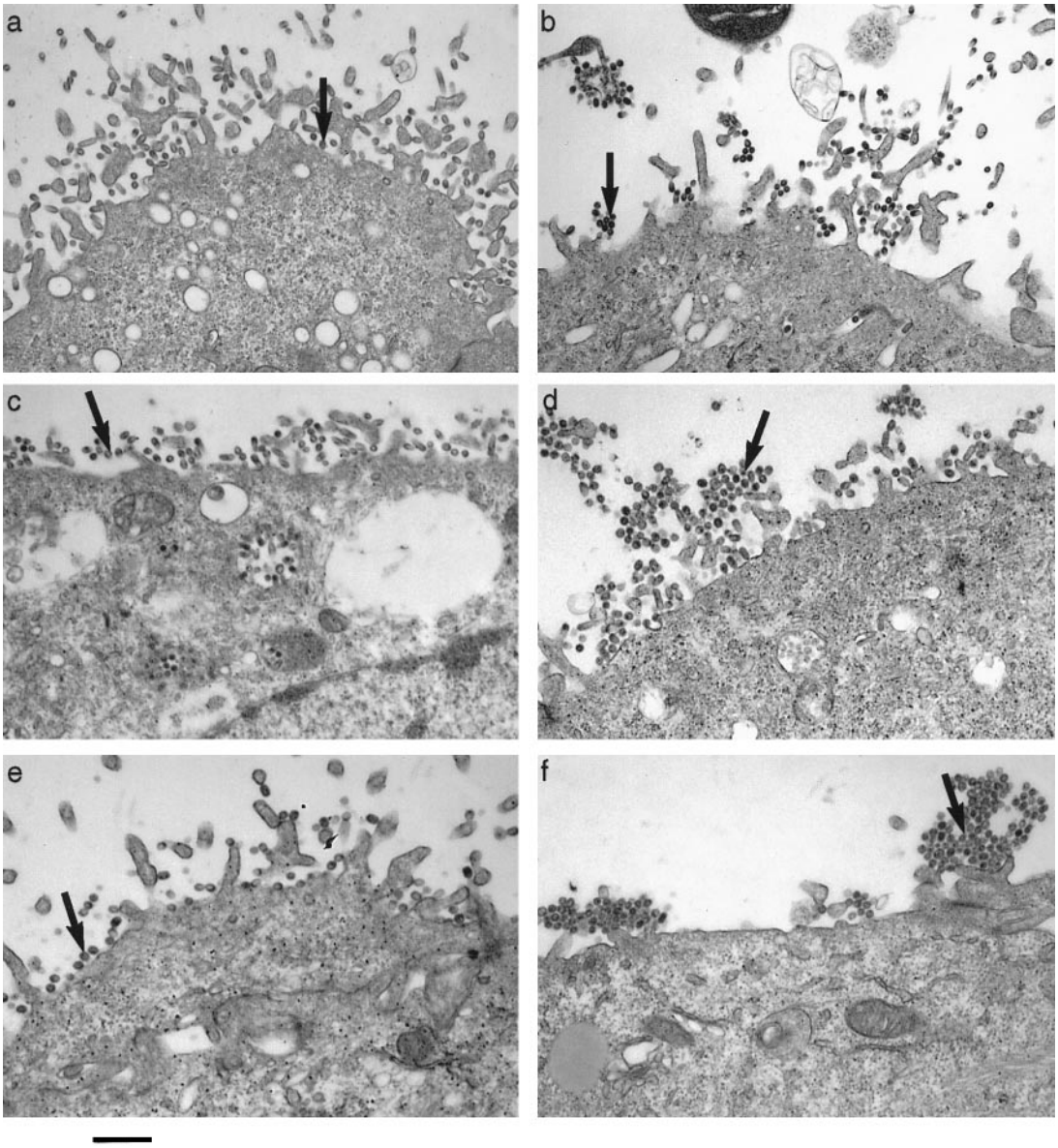


FIG. 2. Electron micrographs of virus budding at the surface of MDCK cells 12 h postinfection (m.o.i. of 5 PFU/cell). (a, c, and e) Nonaggregated virus particles (arrows) budding in zanamivir-free medium. (b, d, and f) Aggregates of virus (arrows) budding in medium containing 1 μM zanamivir. (a and b) SD17. (c and d) Langham. (e and f) Egypt. Marker bar represents 600 nm.

Haemagglutination activity is not significantly affected by zanamivir

Because zanamivir is an analog of sialic acid, the receptor for influenza HA, the effect of zanamivir on haemagglutination was examined. Each of the four viruses agglutinated chicken erythrocytes at 4°C. The effects of zanamivir on haemagglutination were minimal (Table 1), consistent with the fact that by virtue of its structure, zanamivir should not block the receptor binding site of HA. Concentrations of zanamivir up to 100 μM had no effect on haemagglutinating activity of SD17, Langham, or Egypt. The haemagglutination titer of Duck Ireland was decreased twofold by 10 nM zanamivir but was not further reduced by higher concentrations of the drug (data not shown).

Enzymatic activity of all four NAs is sensitive to zanamivir

To examine whether differences in zanamivir sensitivity of replication of the four viruses were due to differences in the zanamivir sensitivity of their NA activities, IC_{50} values for inhibition of NA by zanamivir were determined. IC_{50} values were similar for the NAs of each of the four viruses (Table 1). The IC_{50} values were consistent with those determined for a wide range of human and avian influenza viruses (Gubareva *et al.,* 1995). We ob-

served a greater variation in IC_{90} values, with SD17/Rostock NA being twofold less sensitive to inhibition by zanamivir than Duck Ireland NA (Table 1). However, these differences are unlikely to account for the wide variation in sensitivity of virus replication to zanamivir in tissue culture.

The above results show that differences in sensitivity of HA or NA activities to zanamivir do not contribute to the significant differences in sensitivity of these viruses in tissue culture. More efficient virus replication or properties of the virion that lead to reduced dependence on NA for release from the cell surface could potentially contribute to differential sensitivity of influenza viruses to an NA inhibitor. Thus although NA is the target for zanamivir, any one of the eight influenza genes, particularly the HA gene, or a combination of the genes, could potentially contribute to sensitivity in tissue culture. To analyse which genes defined sensitivity to zanamivir, six families of reassortant viruses were produced between pairs of the four avian viruses, and their sensitivities to the drug were analysed.

Production of reassortant viruses

The SD17 × Langham (SL) family, the SD17 × Egypt (SE) family, and the Langham × Egypt (LE) family showed good reassortment between the eight genes and a wide range of recombinant genotypes. In the Duck Ireland × SD17 (DS) family, Duck Ireland × Langham (DL) family, and Duck Ireland × Egypt (DE) family, genes from Duck Ireland were underrepresented, and in the latter two families, there were few reassortants, probably due to the relatively low titer of Duck Ireland in allantoic fluid available for use in the production of reassortants. Viruses with all of the possible segment 4/segment 6 combinations, with the exception of Egypt segment 4 combined with Duck Ireland segment 6, were identified among the reassortants.

Sensitivity to zanamivir in MDCK cells is determined by HA and NA

A total of 54 viruses, from the six families of reassortants, were examined for sensitivity to zanamivir in plaque assays and virus yield assays, and IC_{50} values were calculated for each assay (Table 2). For any virus, IC_{50} was not related to total virus yield in the absence of zanamivir (data not shown), showing that decreased sensitivity was not attributable to more efficient replication. Within each family of reassortants, the viruses were sorted by genotype for each of the eight RNA segments in turn and the influence of the RNA segment on IC_{50} was examined using the rank sum test. The polypeptides encoded by each RNA segment are given in Table 2.

SD17 × Langham family (Table 2). There were 14 viruses analysed in this category. Segment 4 from Langham conferred greater sensitivity than did segment 4 from SD17/Rostock in the plaque assay ($P = 0.01$) and in the virus yield assay, measured by infectivity titer ($P < 0.001$) or haemagglutination titer ($P < 0.05$). The sensitivity phenotype of virus SL12, which has Langham segment 4 in the SD17 genetic background, shows that Langham segment 4 alone is sufficient to confer the increased sensitivity of Langham compared with SD17 in each of the three assays. No effect of segment 6 was observed. However, in the plaque assay, Langham segments 1 and 2 also segregated with greater sensitivity than did SD17 segments 1 and 2 ($P < 0.05$), and this effect of segment 2 was also seen in the virus yield infectivity titer assay.

SD17 × Egypt family (Table 2). There were 14 viruses analysed in this category. Segment 4 from Egypt conferred greater sensitivity than did segment 4 from SD17/Rostock in the plaque assay ($P < 0.01$) and the virus yield assay, measured by infectivity titer ($P < 0.01$) and by haemagglutination titer ($P < 0.05$). In this latter assay, Egypt segments 6 and 8 were also associated with greater sensitivity ($P < 0.05$). However, the sensitivity phenotype of virus SE3, which has Egypt segment 4 in the SD17 genetic background, shows that Egypt segment 4 is sufficient to confer the increased sensitivity of Egypt compared with SD17. Egypt segment 6 was consistently associated with an increase in sensitivity of viruses with SD17/Rostock segment 4, in each of the three assays.

Langham × Egypt family (Table 2). Twelve viruses were analysed in this family. In the plaque assay, greater sensitivity was associated with Egypt segments 2 ($P < 0.01$), 4 ($P < 0.05$). and 6 ($P = 0.05$). Egypt segment 6 was associated with an increase in sensitivity of viruses with Langham segment 4 (viruses LE17 and LE30). In the virus yield infectivity titer assay, no RNA segment showed any significant association with sensitivity. However, in the virus yield haemagglutination titer assay, Egypt segment 4 was significantly associated with increased sensitivity.

Duck Ireland × SD17 family (Table 2). In this family, 16 viruses were examined. Examination of single gene reassortant viruses, with one gene from Duck Ireland in the SD17 genetic background, indicated that Duck Ireland segments 3 and 5 are not associated with sensitivity in the three assays (viruses D9 and D3, respectively). The rank sum test confirmed that segments 3, 5, 1, and 7 were not significantly associated with sensitivity in any of the assays. Duck Ireland segment 4 was significantly associated with sensitivity in the plaque assay ($P < 0.01$) and in both virus yield assays ($P < 0.05$). The data for virus DS7 show that Duck Ireland segment 4 alone confers some increase in sensitivity in the plaque assay and a great increase in sensitivity in both of the virus yield assays. Duck Ireland segment 6 was associated with large increases in sensitivity in each of the three assays (virus DS17), and this association was significant in the plaque assay ($P < 0.001$) and the virus yield haemagglutination titer assay ($P < 0.05$). The relatively resistant

TABLE 2

Correlation of Genotype with Zanamivir-Sensitivity Phenotype in Three Tissue Culture Assays (MDCK Cells)

	Gene								Assay		
Virus	1 PB2	2 PB1	3 PA	4 HA	5 NP	6 NA	7 M1/M2	8 NS1/NS2	A	B	C
						SL family of reassortants					
SD17	S	S	S	S	S	S	S	S	147	10^4–10^5	178
SL1	S	S	S	S	S	S	S	S	163	10^4–10^5	107
SL10	S	S	S	S	S	S	S	L	327	10^4–10^5	578
SL15	S	S	L	S	S	S	L	L	327	10^4–10^5	594
SL11	S	S	S	S	L	L	S	S	154	10^4–10^5	594
SL24	S	S	S	S	L	L	L	L	402	10^4–10^5	594
SL30	S	S	L	S	L	L	S	S	158	10^4–10^5	135
SL12	S	S	S	L	S	S	S	S	67	105	63
SL20	S	S	L	L	L	S	S	L	26	76	185
SL25	L	L	L	L	S	S	L	L	10	44	133
SL27	S	L	L	L	L	L	L	S	72	23	56
SL35	S	S	L	L	L	L	S	L	82	154	63
SL17	L	L	L	L	L	L	L	L	30	323	133
Langham	L	L	L	L	L	L	L	L	28	304	101
						SE family of reassortants					
SD17	S	S	S	S	S	S	S	S	147	10^4–10^5	178
SE9	S	S	S	S	S	S	S	S	123	10^4–10^5	1000
SE20	S	E	S	S	S	S	S	S	175	10^4–10^5	572
SE22	S	E	S	S	S	S	S	S	266	10^4–10^5	629
SE7	S	S	E	S	E	E	S	S	33	10^3–10^4	38
SE24	E	E	S	S	S	E	E	S	33	755	18
SE15	S	E	S	S	S	E	E	E	3	832	7
SE8	E	E	E	E	E	S	S	E	3	124	10
SE3	S	S	S	E	S	S	S	S	2	15	32
SE13	E	E	E	E	E	S	E	S	3	ND	ND
SE10	E	E	E	E	S	E	S	S	3	1000	6
SE5	E	E	E	E	S	E	S	E	3	389	6
SE25	S	E	S	E	S	E	E	E	3	335	6
Egypt	E	E	E	E	E	E	E	E	3	200	9
						LE family of reassortants					
Langham	L	L	L	L	L	L	L	L	28	304	101
LE1	E	L	L	L	L	L	L	L	28	87	134
LE16	E	L	E	L	L	L	L	E	32	100	34
LE25	E	L	L	L	L	L	E	L	25	60	13
LE17	E	E	E	L	E	E	E	E	3	100	56
LE30	E	E	E	L	E	E	E	E	3	874	56
LE10	E	E	E	E	E	L	E	E	1	42	10
LE12	L	E	E	E	E	L	E	E	1	ND	ND
LE20	L	E	L	E	L	E	E	E	3	247	6
LE18	E	E	E	E	E	E	E	E	3	29	5
LE21	L	L	E	E	E	E	L	E	3	ND	ND
Egypt	E	E	E	E	E	E	E	E	3	200	9

phenotype of DS7 and DS12, in the plaque assay, demonstrates that SD17/Rostock segment 6 can decrease the sensitivity of viruses with Duck Ireland segment 4. Duck Ireland segment 2 showed an association with increased sensitivity in the plaque assay (virus B9) but not in the virus yield assays. Duck Ireland segment 8 segregated with sensitivity only in the plaque assay ($P < 0.01$).

Duck Ireland × Langham family (Table 2). Seven viruses were examined. There were too few reassortants of each genotype to allow statistical analyses to be carried out. However, in the plaque assay, Duck Ireland

TABLE 2—*Continued*

Virus	Gene 1 PB2	2 PB1	3 PA	4 HA	5 NP	6 NA	7 M1/M2	8 NS1/NS2	Assay A	B	C
DS family of reassortants											
SD17	S	S	S	S	S	S	S	S	147	10^4–10^5	178
B15	S	S	S	S	S	S	S	S	132	10^4–10^5	594
C8	S	S	S	S	S	S	S	S	114	10^4–10^5	178
B9	S	D	S	S	S	S	S	S	66	10^4–10^5	134
D9	S	S	D	S	S	S	S	S	175	10^4–10^5	495
D3	S	S	S	S	D	S	S	S	171	10^4–10^5	1000
DS17	S	S	S	S	S	D	S	S	3	4000	8
DS28	S	S	S	S	D	D	S	S	5	2000	8
C15	D	S	D	S	D	D	D	S	7	251	13
DS7	S	S	S	D	S	S	S	S	67	12	7
DS12	S	S	S	D	S	S	S	D	24	ND	ND
D2	S	S	S	D	S	D	S	D	2	21	6
C16	S	D	S	D	S	D	S	D	1	ND	ND
B14	D	D	D	D	D	D	S	D	1	ND	ND
C5	D	D	D	D	D	D	D	D	3	ND	ND
Duck	D	D	D	D	D	D	D	D	1	ND	ND
DL and DE reassortants											
Langham	L	L	L	L	L	L	L	L	28	304	101
DL12	L	L	L	L	L	L	L	L	84	80	132
DL24	L	L	L	L	L	L	L	L	34	122	75
DL69	L	D	L	L	D	D	D	L	4	86	8
DL27	D	L	L	D	D	L	L	L	1	ND	ND
DL64	L	L	L	D	D	L	L	L	2	ND	ND
DE6	E	E	E	D	E	E	D	E	3	ND	ND
Egypt	E	E	E	E	E	E	E	E	3	200	9
Duck	D	D	D	D	D	D	D	D	1	ND	ND

Note. Mean IC_{50} (nM) values are given for three tissue culture assays: A, plaque assay; B, virus yield infectivity titre assay; and C, virus yield haemagglutination titre assay. Genotype of viruses is given by letters indicating the parent virus from which each gene was derived: S, SD17; L, Langham; E, Egypt; and D, Duck Ireland. Viruses have been listed by segment 4 and segment 6 genotype. ND, Not done: not all viruses were available for use at high m.o.i. for the virus yield assay due to low titres of infectious virus in allantoic fluid.

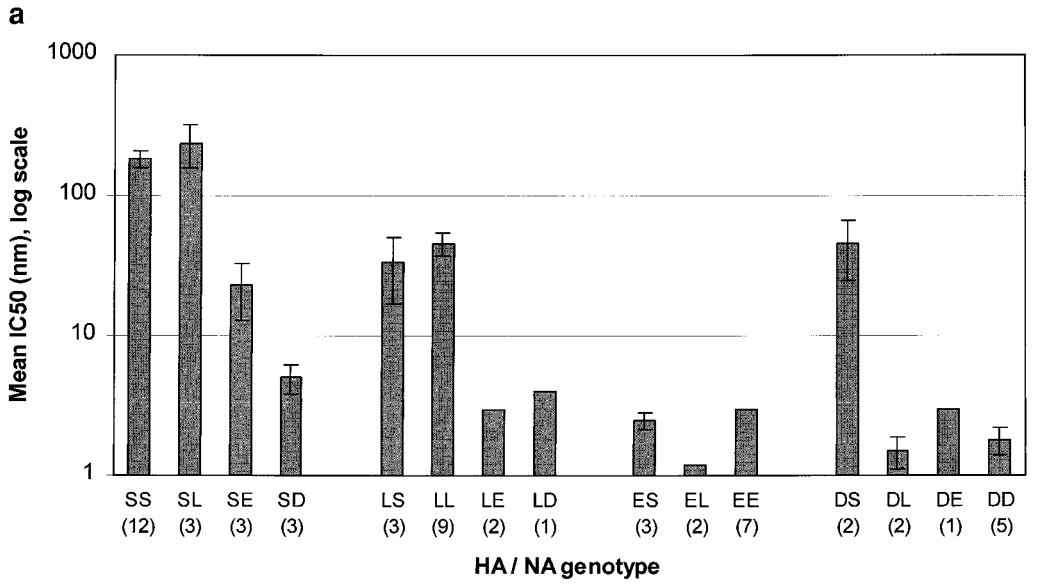
segments 4 and 6 were observed to be associated with greater sensitivity, and in the virus yield haemagglutination titer assay, Duck Ireland segment 6 was associated with increased sensitivity of virus DL69, compared with Langham, DL12, and DL24.

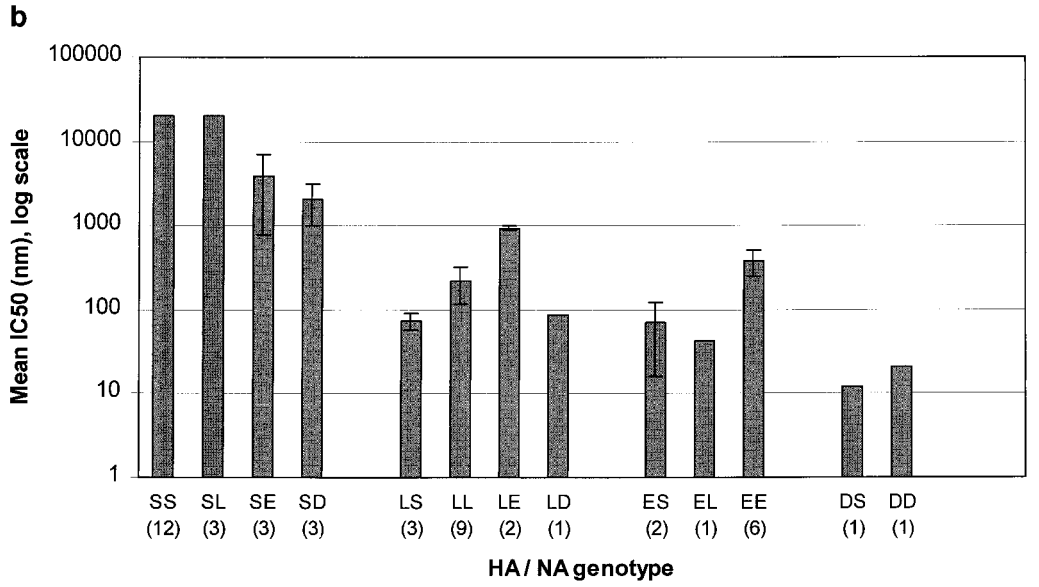
Duck Ireland × Egypt reassortant (Table 2). Only one reassortant (DE6) was produced. This virus was analysed in the plaque assay, where its phenotype did not differ from that of either of the parent viruses.

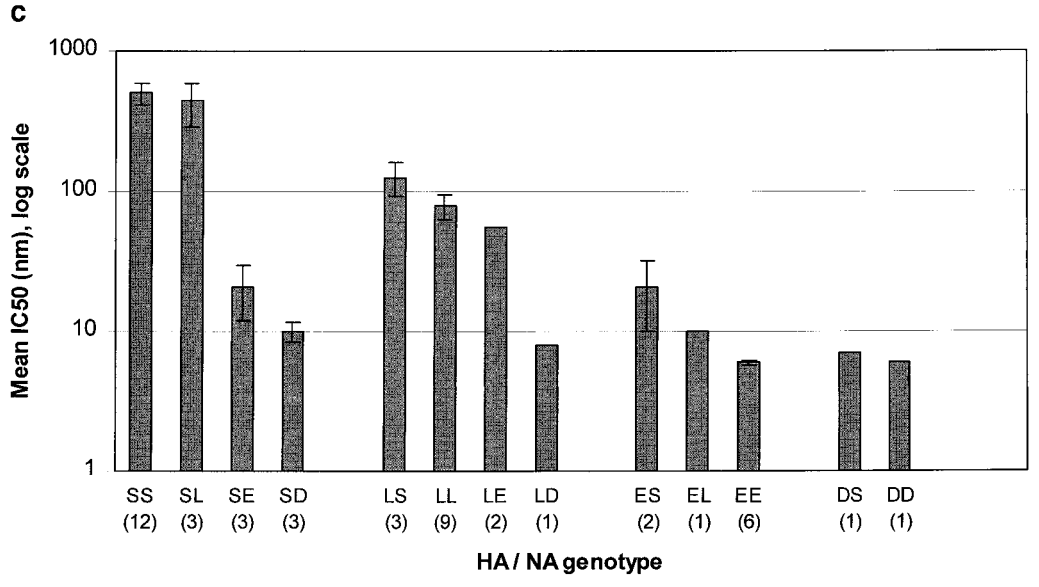
To summarise, sensitivity to zanamivir in tissue culture was consistently determined by segment 4 (HA) and segment 6 (NA). The effect of the segment 4/segment 6 combination on sensitivity is given in Fig. 3. In each assay, the relative contribution of each HA to sensitivity paralleled the relative sensitivity of the parent virus from which the HA was derived, with the SD17/Rostock HA being associated with lowest sensitivity. NA also influenced sensitivity: in the plaque assay and the virus yield haemagglutination titer assay, Egypt NA and Duck Ireland NA clearly conferred greatest sensitivity, whereas SD17/Rostock NA and Langham NA were associated with decreased sensitivity (Fig. 3). An effect of NA on sensitivity of influenza virus replication to FANA was noticed by Palese *et al.* (1974), although the characteristics of these NAs were not investigated. We observed that in certain families of reassortants and in certain assays, segments 1 and/or 2 and/or 8 segregated inconsistently with sensitivity. This could be an indirect effect caused by genetic linkage between these segments and segments 4 or 6 and/or random association caused by the large number of statistical analyses carried out.

Sequence analysis of HA and NA genes from representative reassortant viruses

It was considered possible that during reassortment, viruses with mutations in the HA and/or NA were selected and that these mutations might contribute to differences in sensitivity to zanamivir between reassortant and parent viruses. We therefore sequenced


a
Mean IC50 (nm), log scale
1000
100
10
1
SS (12)
SL (3)
SE (3)
SD (3)
LS (3)
LL (9)
LE (2)
LD (1)
ES (3)
EL (2)
EE (7)
DS (2)
DL (2)
DE (1)
DD (5)
HA / NA genotype


b
Mean IC50 (nm), log scale
100000
10000
1000
100
10
1
SS (12)
SL (3)
SE (3)
SD (3)
LS (3)
LL (9)
LE (2)
LD (1)
ES (2)
EL (1)
EE (6)
DS (1)
DD (1)
HA / NA genotype


c
Mean IC50 (nm), log scale
1000
100
10
1
SS (12)
SL (3)
SE (3)
SD (3)
LS (3)
LL (9)
LE (2)
LD (1)
ES (2)
EL (1)
EE (6)
DS (1)
DD (1)
HA / NA genotype

the HA and/or NA genes of representative reassortant viruses.

Because viruses DS7 (H5) and DS12 (H5) showed a reduced sensitivity to zanamivir compared with Duck Ireland (H5), the HA1 region of the HA genes of DS7, DS12, D2, and DE6 (all H5) were sequenced. None of the viruses showed any nucleotide sequence changes in HA1, compared with Duck Ireland HA1 sequence.

The nucleotide sequences of the NA genes of five reassortants, all with SD17/Rostock NA, were also determined. These viruses were SL10 (SD17/Rostock HA), SL20 (Langham HA), SE8 (Egypt HA), DS7, and DS12 (both Duck Ireland HA). Compared with the NA from SD17 virus, SL10, SE8, DS7, and DS12 showed no nucleotide sequence changes in the NA gene. SL20 has one nucleotide substitution (G→A at position 393), resulting in replacement of glycine by arginine at amino acid residue 125. From the virus characteristics addressed in this work (elution from erythrocytes and sensitivity to zanamivir in tissue culture), there is no evidence to suggest that this mutation has any effect on the properties of the NA of SL20 or the phenotype of the virus.

These sequence data from representative viruses indicate it is highly unlikely that mutations in HA or NA acquired during the reassortment are responsible for the observed differences in sensitivity to zanamivir.

Ability to elute from chicken erythrocytes depends on combination of HA and NA

Because the effects of zanamivir on haemagglutination were minimal, whereas each of the four NA activities was sensitive to zanamivir, we performed haemagglutination-elution studies to further investigate the interactions between HA and NA that may determine sensitivity to zanamivir. Each of the parent and reassortant viruses was able to agglutinate erythrocytes at 4°C, showing that all could bind to the oligosaccharide receptors on the erythrocyte surface. However, on transfer to 37°C, the viruses fell into two groups (Table 3): those that were able to elute from erythrocytes within 7 h (including SD17, Egypt, and Duck Ireland) and those that had not eluted at termination of the assay at 20 h (including Langham). Ability to elute depended on the combination of HA and NA possessed by the virus. All viruses with the same HA/NA combination behaved similarly (Table 3), independent of the origin of their other six genes. Viruses with Duck Ireland, Egypt, or Langham HA could elute only if they possessed Duck Ireland or Egypt NA. Thus Egypt NA and Duck Ireland NA differ from SD17/Rostock NA and Langham NA in a manner that confers the ability to elute more readily. In the presence of exogenous NA [*Clostridium perfringens* NA (CPNA)] at 10 mU/ml, 12 viruses, representative of the six noneluting combinations of HA/NA, eluted fully within 3 h (data not shown), confirming that it is the NA of these viruses (SD17/Rostock NA or Langham NA) that is responsible for their inability to elute naturally. All viruses with SD17/Rostock HA could elute, independent of their NA, so SD17/Rostock HA differs from the other three HAs in some respect that confers the ability to elute more readily. Viruses with the combination of SD17/Rostock HA with either Egypt or Duck Ireland NA eluted most rapidly (Table 3).

TABLE 3

Elution of Influenza Viruses from Chicken Erythrocytes at 37°C

HA type	NA type	Ability to elute	Time taken for elution of 4 HA U (h)	[Zanamivir] (nM) required to inhibit elution of 4 HA U
S (H7)	S(N1)	+[a]	1–7	50–200
	L(N3)	+	3	200–1000
	E(N1)	+	0.5–1	100–500
	D(N8)	+	0.5–1	50–100
L (H7)	S(N1)	−[b]	N/A[d]	N/A
	L(N3)	−	N/A	N/A
	E(N1)	+	3–7	5–10
	D(N8)	+	3	5
E (H7)	S(N1)	−	N/A	N/A
	L(N3)	−	N/A	N/A
	E(N1)	+	1–7	10
	D(N8)	No virus[c]	N/A	N/A
D (H5)	S(N1)	−	N/A	N/A
	L(N3)	−	N/A	N/A
	E(N1)	+	1	20
	D(N8)	+	3–7	10

Note. S, SD17/Rostock; L, Langham; E, Egypt; D, Duck Ireland.
[a] Elution at 37°C
[b] No elution at 37°C.
[c] No virus with this genotype was produced.
[d] N/A, not applicable.

Elution, from chick erythrocytes, of viruses with SD17/Rostock HA is less sensitive to inhibition by zanamivir

The concentration of zanamivir required to inhibit elution of 4 HA units virus was recorded at regular intervals after transfer of the plates to 37°C. The lowest concentration of zanamivir that totally inhibited elution at 16 h was recorded (Table 3) because, in the absence of zana-

FIG. 3. Effect of HA and NA combination on sensitivity of replication of avian influenza viruses to zanamivir in MDCK cells in three assays: plaque number (a), virus yield measured by infectivity titer on CEF cells (b), and virus yield measured by haemagglutination titer (c). Viruses are grouped by HA and NA type, and mean IC_{50} values for each group are given. Error bars indicate SEM. The origin of the HA and NA, and the number of viruses examined in each group, are given below the bars. S, SD17/Rostock HA or NA; L, Langham HA or NA; E, Egypt HA or NA; D, Duck Ireland HA or NA.

mivir, the viruses eluted at different rates and it was not appropriate to compare sensitivity of viruses at an early timepoint. The elution from erythrocytes of all viruses with SD17/Rostock HA, independent of their NA, was less sensitive to inhibition by zanamivir than was the elution of viruses with one of the other three HAs.

Because the combination of HA and NA determines both sensitivity of virus to zanamivir in tissue culture and the ability of viruses to elute from erythrocytes, we further examined the properties of the four NAs and four HAs that could influence ability to elute.

All four avian virus NAs preferentially cleave 2,3-*N*-acetylneuraminyl-lactose

Because compatibility between HA receptor binding specificity and NA substrate cleavage specificity is a prerequisite for elution from erythrocytes (Carroll *et al.,* 1981; Baum and Paulson, 1991; Gimsa *et al.,* 1996), we investigated the substrate specificity of the NAs. The human control virus (X-31) released sialic acid from both 2,3- and 2,6-*N*-acetylneuraminyl-lactose, with a slight preference for the former substrate (Fig. 4). Each of the four avian virus NAs exhibited a strong preference for 2,3-*N*-acetylneuraminyl-lactose as a substrate over 2,6-*N*-acetylneuraminyl-lactose (Fig. 4), so differences in elution properties cannot be attributed to differences in NA cleavage specificity. Additionally, horse serum, which contains sialic acid in 2,6-Gal linkages (Rogers *et al.,* 1983), did not inhibit haemagglutination by any of the viruses (data not shown), indicating that each of these viruses does not bind appreciably to Neu5AcAlpha2–6Gal and therefore, by implication, they bind preferentially to Neu5AcAlpha2–3Gal. Moreover, in each of the viruses, the HA contains a glutamine at residue 226 (H3 numbering), this glutamine being associated with Neu5AcAlpha2–3Gal binding specificity (Rogers *et al.,* 1983).

Deletions in the NA stalk region are associated with inability of virus to elute from erythrocytes

It has previously been reported that viruses with deletions in the NA stalk show a reduced ability to elute from chicken erythrocytes (Els *et al.,* 1985; Castrucci and Kawaoka, 1993) and are deficient in their ability to cleave fetuin (Els *et al.,* 1985). We therefore determined the sequence of the stalk region of each of the four avian virus NAs. The amino acid sequences of the NAs, from the amino terminus to the end of the stalk, are aligned in Fig. 5. The amino acids composing the stalk are underlined. The stalk regions are taken to begin at His or Lys (potentially charged amino acid residues) that mark the end of the 29- 31-amino-acid hydrophobic region. Cys90, in the N8 sequence, and the equivalent Cys in the other three sequences, were taken as the first amino acid in the head region (Blok and Air, 1982a,b). Egypt NA and

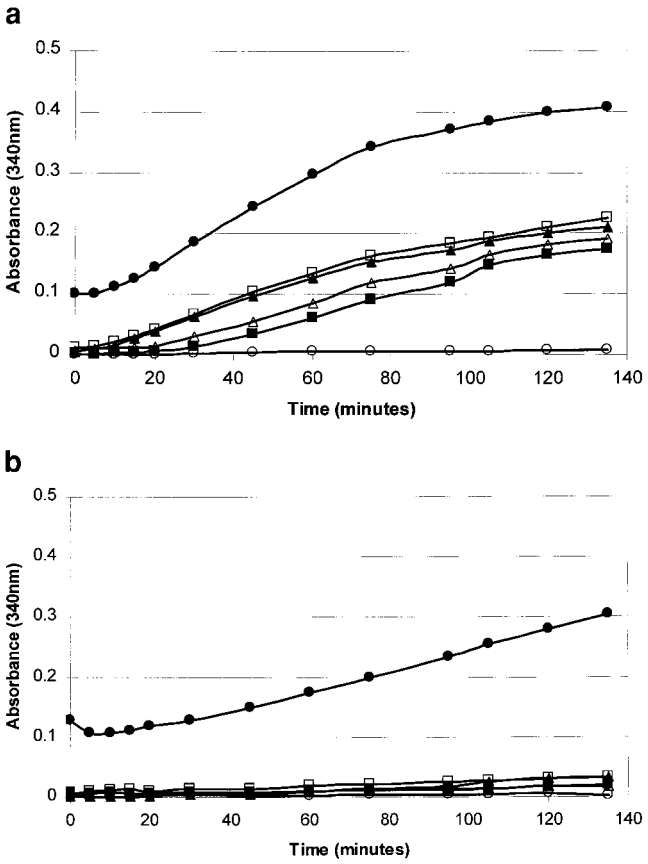


FIG. 4. NA activities of purified avian influenza viruses using 2,3-*N*-acetyl neuraminyl-lactose (a) and 2,6-*N*-acetyl neuraminyl-lactose (b) as substrates. NA activity, for a constant amount of protein from purified virus, was measured using a coupled assay method releasing NADH, the absorbance of which is recorded at 340 nm. Viruses examined were SD17 (△), Langham (■), Egypt (▲), Duck Ireland (□), and X-31 (●). The control sample (no virus) is indicated by ○.

Duck Ireland NA both have long stalks (lengths 56 and 52 amino acids, respectively). SD17/Rostock NA (N1) and Langham NA (N3) each have a deletion in the stalk region (Fig. 5), resulting in short stalks of lengths of 34 and 28 amino acids, respectively. Furthermore the rate of cleavage of 2,3-*N*-acetylneuraminyl-lactose, assayed for a constant amount of protein from purified virus, was greater for Egypt NA and Duck Ireland NA than for SD17/Rostock NA and Langham NA (Fig. 4a).

To summarise, consistent with the results of Els *et al.* (1985) and Castrucci and Kawaoka (1993), the two avian influenza virus NAs that were able to promote elution (Egypt and Duck Ireland NAs) were those with long stalks and a greater rate of substrate cleavage, whereas the two NAs that were unable to promote elution (SD17/Rostock and Langham NAs) were those with short stalks and a lower rate of substrate cleavage. It is likely that release of virus from its receptors and disaggregation of virus particles by short-stalk NAs are inefficient because the NA active site is close to the virus envelope and therefore cannot efficiently access its substrate.

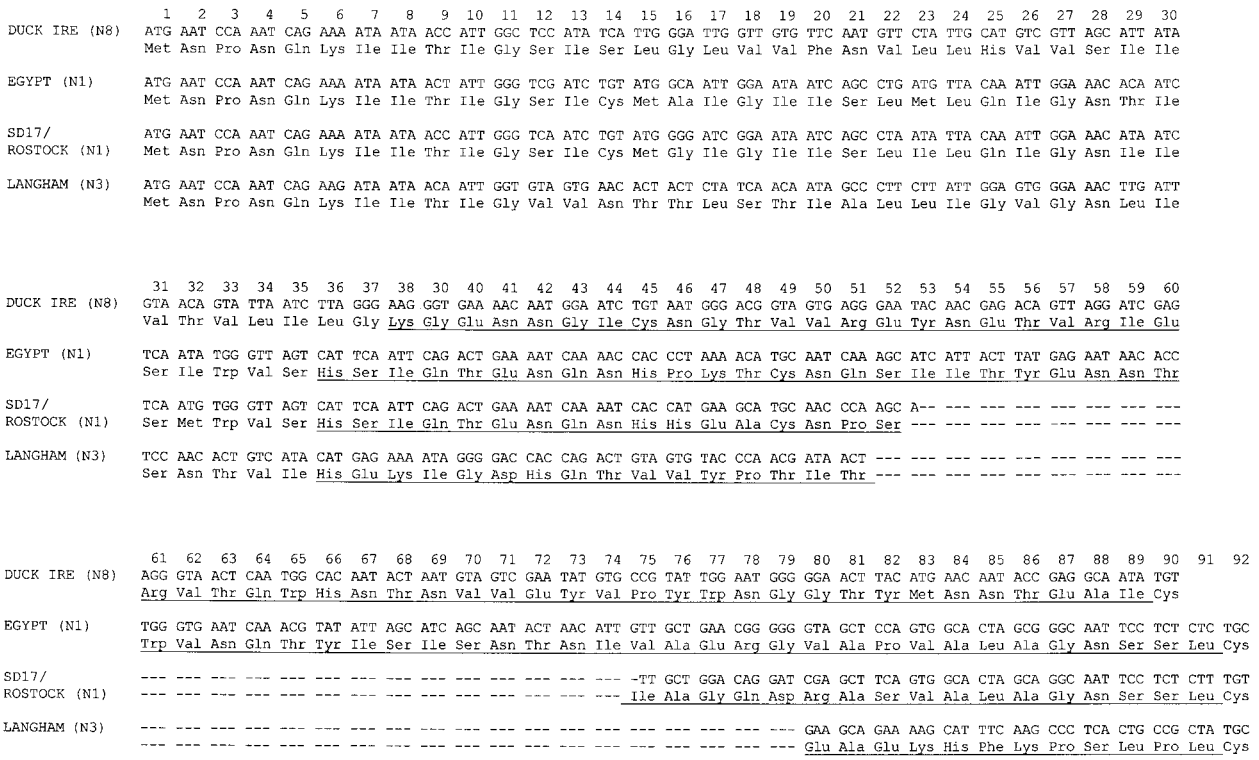


FIG. 5. Comparison of nucleotide and amino acid sequences of NAs from the four avian influenza viruses. Sequences are aligned from the N-terminus to the end of the stalk region. The amino acids making up the stalk are underlined. The locations of deletions are indicated by dashes.

SD17/Rostock HA shows reduced dependence on NA for elution from its receptor

Because viruses with SD17/Rostock HA could elute, even when they possessed a short-stalk NA, and elution of viruses with SD17/Rostock HA was least sensitive to zanamivir, we hypothesised that the HA molecule of SD17/Rostock can elute from its receptors with decreased dependence on NA activity, circumventing zanamivir-induced inhibition of NA. The nature and extent of glycosylation of HA have been implicated in altering the affinity or availability of HA molecules for their receptors (Mir-Shekari *et al.,* 1997) and in resistance to zanamivir (Staschke *et al.,* 1995). The presence of glycans close to the receptor binding site of HA of A/FPV/Rostock/34, at Asn123 and Asn149 (H7 numbering) (Kiel *et al.,* 1985), is essential for release of this virus from cellular receptors (Ohuchi *et al.,* 1997), and receptor binding is abolished when these glycans are sialylated after expression in the absence of NA (Ohuchi *et al.,* 1995). We therefore determined the locations of potential glycosylation sites on the HA trimers of our other three avian influenza viruses from the amino acid sequences of these molecules. The location of the glycosylation sites (Fig. 6) differs both between and within HA subtypes. At the distal tip, SD17/Rostock HA (H7) has two glycans attached to Asn123 (equivalent to 133 in H3 numbering) and Asn149 (158 in H3 numbering). The HAs of Langham (H7) and Egypt (H7) possess a glycosylation site at position 123, and Duck Ireland (H5) has a single potential glycosylation site at the distal tip of the HA, at residue 175 (169 H3 numbering), as deduced from the nucleotide sequence (Kawaoka *et al.,* 1987). Because glycosylation is absent at position 149 in the HA molecules of Langham and Egypt and at the equivalent position in the HA of Duck Ireland, we suggest that the glycan at position 149 of SD17/Rostock HA allows SD17 to elute from its receptors with decreased dependence on NA activity. This is likely to be because the oligosaccharide side chain reduces the affinity of HA for its receptor by steric hindrance. An alternative explanation is that sialic acid bound to position 149 of SD17/Rostock HA may fill the receptor binding pocket, competing with sialic acid attached to cellular receptors or to virus particles. However, we cannot formally exclude the possibility that decreased affinity of SD17/Rostock HA for receptors is due to differences in amino acid sequence at the receptor binding site, compared with the HAs of Langham, Egypt, and Duck Ireland.

Thus elution from erythrocytes requires a long-stalked NA and/or a more readily released HA that may compensate for the inefficient function of a short-stalked NA and tip the balance in favour of elution; these observations are supported by recent data from Matrosovich *et al.* (1999). Possession of Egypt NA and Duck Ireland NA (long-stalked NAs) also correlates with greatest sensitivity in tissue culture assays, whereas SD17/Rostock HA (the readily released HA) correlates with lowest sensi-

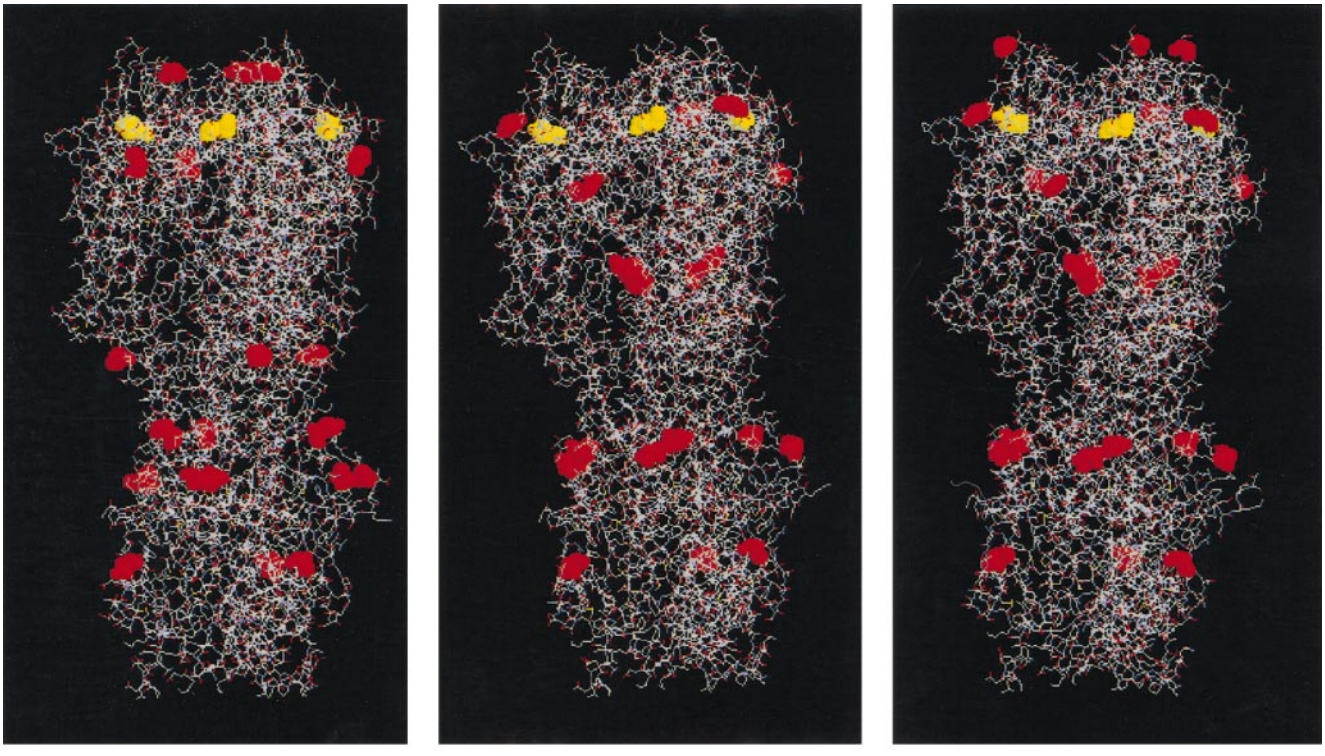


FIG. 6. Position of potential glycosylation sites on the HA trimers of Duck Ireland, H5 (A), Langham, H7 and Egypt, H7 (B), and SD17/Rostock, H7 (C) shown on a model of the H3 HA (Brookhaven protein databank). Potential sites to which carbohydrate moieties are attached are space-filled in red, and the receptor binding pocket at the top of each HA monomer is marked by residue Leu226 (H3 numbering), space-filled in yellow. The NXT/S motifs are located at the following positions (H3 numbering): Duck Ireland HA1: 20, 21, 33, 169, 197, 289; HA2: 154. Langham and Egypt HA1: 22, 38, 133, 240; HA2: 82, 154; Rostock HA1: 22, 38, 133, 158, 240; and HA2: 82, 154.

tivity. Having established the features of HA and NA that are associated with ability of our viruses to elute from erythrocytes, we can begin to interpret the differences in zanamivir-sensitivity of the four avian influenza viruses in tissue culture. The relative resistance of SD17 can be explained by the characteristics of its HA, which enables the virus to be released from cells with decreased dependence on NA. We suggest that if a virus possesses SD17/Rostock HA in combination with a short-stalked NA (SD17/Rostock NA or Langham NA), the virus is relatively resistant to zanamivir *in vitro* because the release of virus from cells is effected by the HA and the short-stalked NA plays only a minor role. However, if a virus possesses the SD17/Rostock HA in combination with a long-stalked NA (Egypt NA or Duck Ireland NA), sensitivity to zanamivir is significantly increased because a long-stalked NA plays a greater role in the release of virus from cells.

Viruses with Langham HA bind more stably to erythrocytes than do viruses with SD17/Rostock HA. Hence we hypothesise that Langham is more sensitive to zanamivir because it is more dependent on NA for release from cells. In the plaque assay, where the effect is amplified by multiple rounds of virus replication, an NA with a long stalk plays a greater role in release of virus from cells than does an NA with a short stalk.

All viruses with Egypt HA show sensitivity to zanamivir in tissue culture. We suggest that Egypt HA binds very stably to MDCK cells in culture, so that all viruses with Egypt HA are dependent on NA for their release from cells, whether they possess a short- or a long-stalked NA.

Most viruses with Duck Ireland HA are highly dependent on NA for release and are highly sensitive to zanamivir, indicating that this HA also binds efficiently to MDCK cells. However, viruses with Duck Ireland HA in combination with SD17/Rostock NA (H5N1) were significantly less sensitive to zanamivir in the plaque assay than all other viruses with Duck Ireland HA. This reduction in sensitivity was not associated with nucleotide changes in the coding sequence of HA1 or NA, compared with the parent viruses from which segment 4 and 6 were derived, and was not associated with any differences in the specific activity of NA between these viruses (data not shown). However, it is possible that differences in stability of NAs can influence virus sensitivity to zanamivir in tissue culture. The long-stalked Egypt and Duck Ireland NAs showed a significantly

greater decrease in activity, after 1-h incubation at 55°C, than did the SD17 and Langham NAs (unpublished observations). It is therefore possible that during the course of the plaque assays, there is a decrease in NA activity in viruses with Egypt or Duck Ireland NA, and sensitivity of these viruses to zanamivir would be increased. The combination of Duck Ireland HA with SD17/Rostock NA might be particularly favourable to replication in tissue culture due to the increased stability of the NA.

Like the H5 HA of A/Duck/Ireland/113/83, the H5 HA of A/HongKong/156/97 (the avian influenza virus recently isolated from humans) lacks the additional glycosylation site at the tip of the molecule (Matrosovich *et al.,* 1999). Also, the N1 NA of A/HongKong/156/97 has a short stalk (Claas *et al.,* 1998; Bender *et al.,* 1999) similar in length to that of the N1 NA of SD17/Rostock. Therefore, we would expect our H5N1 viruses to have a similar zanamivir-sensitivity phenotype to A/HongKong/156/97. This latter virus has an IC_{50} value of 1 nM in plaque assays (Gubareva *et al.,* 1998) and thus would appear to be more sensitive than our DS7 and DS12 viruses. However, we used plaque number assays to determine virus sensitivity to zanamivir, whereas Gubareva *et al.* (1998) used plaque size reduction assays. This factor, together with possible differences in MDCK cells, could contribute to the reported differences in sensitivity to zanamivir.

It has previously been shown that changes in affinity of HA for specific sialic acid receptors, influencing ability of the virus to disaggregate and to elute from the cell surface, can overcome NA deficiency or inhibition in NA-deficient mutants (Yang *et al.,* 1997) in laboratory-produced avian-human reassortant influenza viruses (Kaverin *et al.,* 1998) and in artificially selected zanamivir-resistant variants (McKimm-Breschkin *et al.,* 1996; Gubareva *et al.,* 1996). Our results show that the naturally occurring "resistance" of SD17 to zanamivir also depends on an HA-related decrease in dependence on NA for virus release. Our study also indicates a role for NA stalk length in determining sensitivity to zanamivir and shows that the level of sensitivity is determined by the combination of HA and NA genotypes. It is pertinent that a synergistic interaction of HA and NA mutations in influencing sensitivity to the drug has also been demonstrated in zanamivir-resistant mutants selected *in vitro* (Blick *et al.,* 1998).

For any naturally occurring strain of influenza virus, a knowledge of the NA stalk length and the affinity of the HA for its receptor should enable prediction of the sensitivity of virus replication to zanamivir *in vitro*. Sensitivity of viruses to zanamivir *in vitro* has been shown not to be necessarily predictive of sensitivity *in vivo* (Woods *et al.,* 1993). Nevertheless, the knowledge of virus properties, which influence sensitivity in any assay system, provides a further understanding of the characteristics of new viruses that may emerge on the widespread use of this inhibitor.

MATERIALS AND METHODS

Viruses

All influenza viruses were from a collection held at The Institute for Animal Health. Four avian influenza viruses were used: A/Duck/Ireland/113/83, H5N8 (Duck Ireland); A/FPV/Egypt/45, H7N1 (Egypt); A/FPV/England/1/63, H7N3 (Langham); and SD17 (H7N1), a reassortant virus with RNA segments 4, 6, and 7 from A/FPV/Rostock/34 (H7N1) and the remaining five segments from A/FPV/Dutch "Dobson"/27 (H7N7). The human influenza A virus X-31 (H3N2) is a reassortant between A/Aichi/1/68 and A/Puerto Rico/8/34. Viruses were propagated in 11-day-old embryonated hens' eggs, at 37°C.

Cells

MDCK cells were maintained in Eagle's modified minimal essential medium (EMEM, GIBCO BRL) containing 0.14% BSA and supplemented with glutamine and antibiotics. Primary chick embryo fibroblast (CEF) cells were prepared from 10-day-old chick embryos and were maintained in M199 medium (GIBCO BRL) containing 0.14% BSA and supplemented with glutamine and antibiotics.

NA inhibitor

Zanamivir was provided by Glaxo Wellcome Research and Development (UK).

Generation, selection, and genotyping of reassortant viruses

Reassortant viruses were isolated after single-cycle mixed infection of CEF cells with pairs of avian influenza viruses. The medium was harvested after 18 h, and reassortant viruses were purified by two rounds of plaque purification in CEF cells and then inoculated into embryonated eggs. Reassortant viruses were genotyped by serological analysis, by electrophoretic analysis of virus polypeptides, and by nucleic acid analysis. Where HAs were distinguishable using antisera, the HA of reassortants was determined by HA-inhibition assays. Where NAs were distinguishable using antisera, the NA of reassortants was determined by NA-inhibition assays (van Deusen *et al.,* 1983). One antiserum was produced in our laboratory, and the remainder were provided by the Central Veterinary Laboratory (Weybridge, UK). Electrophoretic polypeptide analysis (as described by McCauley and Penn, 1990) was used to determine the genotype of segments 4, 6, and 8 for all reassortants and segments 1 and/or 2 and/or 3 for some reassortant viruses. For nucleic acid analysis, virus-specific oligonucleotides were designed for RNA segments 1–3, 5, and 7. These were used to determine genotype of reassortants by either PCR or by dot-blot hybridisation. Infected cell RNA was prepared for each reassortant virus. Full-length cDNA was synthesised from the vRNA by avian myelo-

blastosis virus reverse transcriptase (Promega) using segment-specific primers complementary to the nucleotides 8–32 of the vRNA. cDNA was amplified by PCR using a virus-specific oligonucleotide primer and a non-virus-specific oligonucleotide primer. The annealing stages of PCR were carried out at a temperature at which only the homologous primer would bind efficiently to the cDNA. The PCR products were analysed on a 1% agarose gel. Dot-blot hybridisation was also used for genotype analysis. For each reassortant virus, a portion of the RNA segment of interest was amplified from infected cell RNA using RT-PCR. DNA was bound to Hybond-N membrane (Amersham) and probed with ^{32}P-labeled virus-specific oligonucleotides at defined temperatures and analysed by autoradiography.

Haemagglutination and haemagglutination-elution assays

For haemagglutination assays, virus and zanamivir were preincubated for 30 min at room temperature in a volume of 50 μl in PBS. An equal volume of chicken erythrocytes was added to a final concentration 0.5%, and the plates were incubated at 4°C for 1 h. For elution assays, the plates were transferred to 37°C (time 0), and elution was monitored at intervals, by appearance of erythrocyte pellets, up to a time of 20 h. The time taken for elution of 4 HA units virus, in the presence or absence of zanamivir, was recorded. To examine the effect of CPNA (Sigma) on elution, haemagglutination was performed as described above in 2-(*N*-morpholino)ethanesulfonic acid buffer (50 mM, pH 6.5) containing 20 mM Ca^{2+} and 100 mM Na^{+}. CPNA (final concentration 10 mU/ml) was added to the agglutinated erythrocytes, and the plates were incubated at 37°C.

NA inhibition assays

NA activity was assayed by the colourimetric assay of Aymard-Henry *et al.* (1973) in a 100-μl volume in PBS using fetuin as a substrate. Virus was diluted in PBS to give a standard level of enzyme activity (0.2–0.4) measured by A_{549nm} of the chromophore. Infectious allantoic fluid was incubated with zanamivir (3 nM to 1 μM) for 1 h at room temperature and then with fetuin for 30 min at 37°C. The assays were performed on two occasions, in triplicate on each occasion. Mean IC_{50} and IC_{90} values (concentrations of zanamivir required to decrease the enzyme activity by 50% and 90%, respectively) were determined using the method of moving averages (Thompson, 1947).

NA substrate specificity assays

Viruses were purified from infectious allantoic fluid by sucrose gradient centrifugation and resuspended in PBS, and the protein concentration in each sample was determined by the Pierce Coomassie Plus protein assay, after disruption in 8 M urea. A constant amount of protein from purified virus was used as the source of NA to assay cleavage of 2,3-*N*-acetylneuraminyl-lactose or 2,6-*N*-acetylneuraminyl-lactose substrates (Sigma). NA activity was measured using a coupled assay resulting in the release of NADH, the absorbance of which is recorded at 340 nm. The assays were carried out in 32.5 mM 2-(*N*-morpholino)ethanesulfonic acid buffer (pH 6.5) in a final volume of 1 ml. The reaction mixture contained 4 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NAD^{+}, 2.25 U of β-galactosidase, 10 U of glucose dehydrogenase, 0.05 mM substrate, and 2 μg of purified virus protein in PBS. The samples were incubated at 37°C, and A_{340nm} readings were taken up to 120 min. The human influenza virus X-31 and NAs from *C. perfringens* (CPNA, Sigma) and *Arthrobacter ureafaciens* (AUNA, BioWhittaker) were used as controls.

Virus plaque reduction assays

Plaque assays were performed in MDCK cells in EMEM without trypsin. Before the addition of virus inoculum, the cells were incubated at room temperature for 10 min with zanamivir (0.1 nM to10 μM in PBS–0.1% gelatin). The cells were then inoculated with virus, diluted in PBS–gelatin to give 10–100 plaques/well, and incubated at room temperature for 45 min. The virus inoculum and zanamivir were removed, and the cells were overlaid with EMEM, with or without zanamivir (0.1 nM to 10 μM), containing 1% agarose. After incubation at 37°C for 2.5 days, the cells were fixed and stained using PBS containing toluidine blue (0.25% w/v) and formaldehyde (4% v/v). The assays were performed on at least two separate occasions, in duplicate on each occasion. IC_{50} value was determined using the method of moving averages (Thompson, 1947).

Virus yield assays

MDCK cells were inoculated with virus at an m.o.i. of 3–5 PFU/cell. After incubation at room temperature for 45 min, the inoculum was removed and medium [EMEM containing 0.14% BSA, with or without zanamivir (10 nM to 10 μM)] was added. After incubation at 37°C for 3 h, the medium was removed, the cells were washed three times with fresh medium of the same composition to remove any unbound input virus, and fresh medium of the same composition was again added. The medium was harvested after 12 h at 37°C, and virus yield was measured by both plaque assay in CEF cells (infectivity titer) and haemagglutination titer. The assays were performed on two separate occasions, in duplicate on each occasion. IC_{50} was determined using the method of moving averages (Thompson, 1947).

Transmission electron microscopy

To examine the effect of zanamivir on virus budding, confluent monolayers of MDCK cells on 15-cm dishes were inoculated with virus at an m.o.i. of 5 PFU/cell. After 1-h absorption at room temperature, the inoculum was removed, and the cells were washed once and then incubated with fresh EMEM with or without 1 μM zanamivir at 37°C for 9 h. The cells were fixed in cacodylate-buffered 2.5% glutaraldehyde, postfixed in 1% of 0.1 M phosphate-buffered osmium tetroxide, and resuspended in 1% aqueous uranyl acetate. The samples were embedded in 1% agar, dehydrated through a methanol gradient, incubated in propylene oxide, and then embedded in Araldite resin. Sections of 90 nm were cut using an Ultracut E ultramicrotome (Reichart-Jung), collected onto 3-mm-diameter copper Athene grids, and stained using lead citrate. Sections were examined using a Philips EM 300 transmission electron microscope at an accelerating voltage of 80 kV.

Sequencing of virus HA and NA genes

Total cell RNA was prepared from infected CEF cells. Segment 4 or segment 6 cDNA was synthesised from the vRNA by avian myeloblastosis virus reverse transcriptase (Promega) using segment 4 or segment 6 subtype-specific primers complementary to nucleotides 8–32 of the vRNA. The regions to be sequenced were amplified by PCR using *Taq* DNA polymerase with subtype-specific primers. The PCR products were gel-purified using the Wizard DNA purification system (Promega) and sequenced by the dideoxy method with ^{33}-P labeled primers. Some of the HA gene sequences were generated by direct sequencing of vRNA (McCauley and Penn, 1990).

Statistical analyses

For each tissue culture assay, viruses were ranked according to their IC_{50} values, and the effect of genotype on sensitivity to zanamivir was analysed using the rank sum test (Wilcoxon, 1945).

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REFERENCES

Aymard-Henry, M., Coleman, M. T., Dowdle, W. R., Laver, W. G., Schild, G. C., and Webster, R. G. (1973). Influenza virus neuraminidase and neuraminidase inhibition procedures. *Bull. WHO* **48,** 199–202.

Baum, L. G., and Paulson, J. C. (1991). The N2 neuraminidase of human influenza viruses has acquired a substrate specificity complimentary to the haemagglutinin receptor specificity. *Virology* **180,** 10–15.

Bender, C., Hall, H., Huang, J., Klimov, A., Cox, N., Hay, A., Gregory, V., Cameron, K., Lim, W., and Subbarao, K. (1999). Characterisation of the surface proteins of influenza A (H5N1) viruses isolated from humans in 1997–1998. *Virology* **254,** 115–123.

Blick, T. J., Sahasrabudhe, A., McDonald, M., Owens, I. J., Morley, P. J., Fenton, R. J., and McKimm-Breschkin, J. (1998). The interaction of neuraminidase and haemagglutinin mutations in influenza virus in resistance to 4-guanidino-Neu5Ac2en. *Virology* **246,** 95–103.

Blick, T. J., Tiong, T., Sahasrabudhe, A., Varghese, J. N., Colman, P. M., Hart, G. J., Bethell, R. C., and McKimm-Breschkin, J. (1995). Generation and characterisation of an influenza virus neuraminidase variant with decreased sensitivity to the neuraminidase-specific inhibitor 4-guanidino-Neu5Ac2en. *Virology* **214,** 475–484.

Blok, J., and Air, G. M. (1982a). Block deletions in the neuraminidase gene from some influenza viruses of the N1 subtype. *Virology* **118,** 229–234.

Blok, J., and Air, G. M. (1982b). Variation in the membrane-insertion and "stalk" sequences in eight subtypes of influenza type A virus neuraminidase. *Biochemistry* **21,** 4001–4007.

Carroll, S. M., Higa, H. H., and Paulson, J. C. (1981). Different cell-surface receptor determinants of antigenically similar influenza virus haemagglutinins. *J. Biol. Chem.* **256**, 8357–8363.

Castrucci, M. R., and Kawaoka Y. (1993). Biologic importance of neuraminidase stalk length in influenza A virus. *J. Virol.* **67**, 759–764.

Claas, E. C. J., Osterhaus, A. D. M. E., van Beek, R., De Jong, J. C., Rimmelzwaan G. F., Senne, D. A., Krauss, S., Shortridge, K. F., and Webster, R. G. (1998). Human influenza A virus (H5N1) related to a highly pathogenic avian influenza virus. *Lancet* **351,** 472–477.

Els, M. C., Air, G. M., Murti, K. G., Webster, R. G., and Laver, W. G. (1985). An 18-amino acid deletion in an influenza neuraminidase. *Virology* **142,** 241–247.

Gimsa, U., Grotzinger, I., and Gimsa, J. (1996). Two evolutionary strategies of influenza viruses to escape host non-specific inhibitors: Alteration of haemagglutinin or neuraminidase activity. *Virus Res.* **42,** 127–135.

Gubareva, L. V., Bethell, R., Hart, G. J., Murti, K. G., Penn, C. R., and Webster, R. G. (1996). Characterisation of mutants of influenza A virus selected with the neuraminidase inhibitor 4-guanidino-Neu5Ac2en. *J. Virol.* **70**, 1818–1827.

Gubareva, L. V., McCullers, J. A., Bethell, R. C., and Webster, R. G. (1998). Characterisation of influenza A/HongKong/156/97 (H5N1) virus in a mouse model and protective effect of zanamivir on H5N1 infection in mice. *J. Infect. Dis.* **178,** 1592–1596.

Gubareva, L. V., Penn, C. R., and Webster, R. G. (1995). Inhibition of replication of avian influenza viruses by the neuraminidase inhibitor 4-guanidino-2,4-dideoxy-2,3-dehydro-N-acetyl neuraminic acid. *Virology* **212,** 323–330.

Kaverin, N. V., Gambaryan, A. S., Bovin, N. V., Rudneva, I. A, Shilov, A. A., Khodova, O. M., Varich, N. L., Sinitsin, B. V., Makarova, N. V., and Kropotkina, E. A. (1998). Post-reassortment changes in influenza A virus haemagglutinin restoring HA-NA functional match. *Virology* **244,** 315–321.

Kawaoka, Y., Nestorowicz, A., Alexander, D. J., and Webster, R. G. (1987). Molecular analyses of the hemagglutinin genes of H5 influenza viruses: Origin of a virulent turkey strain. *Virology* **158,** 218–227.

Kiel, W., Geyer, R., Dabrowski, J., Dabrowski, U., Niemann, H., Stirm, S., and Klenk, H.-D. (1985). Carbohydrates of influenza virus: Structural elucidation of the individual glycans of the FPV haemagglutinin by two-dimensional ^{1}H n.m.r. and methylation analysis. *EMBO J.* **4,** 2711–2720.

Matrosovich, M., Zhou, N., Kawaoka, Y., and Webster, R. (1999). The surface glycoproteins of H5 influenza viruses isolated from humans, chickens and wild aquatic birds have distinguishable properties. *J. Virol.* **73**, 1146–1155.

McCauley, J. W., and Penn, C. R. (1990). The critical cut-off temperature of avian influenza viruses. *Virus Res.* **17,** 191–198.

McCauley, J. W., Pullen, L. A., Forsyth, M., Penn, C. R., and Thomas, G. P. (1995). Zanamivir fails to protect chickens from infection with highly pathogenic avian influenza viruses. *Antiviral Res.* **27,** 179–186.

McKimm-Breschkin, J. L., Blick, T. J., Sahasrabudhe, A., Tiong, T., Marshall, D., Hart, G., Bethell, R. C., and Penn, C. R. (1996). Generation and characterisation of variants of NWS/G70C influenza virus after *in vitro* passage in 4-amino-Neu5Ac2en and 4-guanidino-Neu5Ac2en. *Antimicrob. Agents Chemother.* **40**, 40–46.

Meindl, P., Bodo, G., Lindner, J., and Palese, P. (1971). Influence of 2-deoxy-2,3-dehydro-N-acetlyneuraminic acid on myxovirus neuraminidases and the replication of influenza and Newcastle disease virus. *Zeitschrift Naturforschung* **26**, 792–797.

Mir-Shekari, S. Y., Ashford, D. A., Harvey, D. J., Dwek, R. A., and Schulze, I. T. (1997). The glycosylation of the influenza A virus haemagglutinin by mammalian cells. *J. Biol. Chem.* **272**, 4027–4036.

Ohuchi, M., Feldmann, A., Ohuchi, R., and Klenk, H.-D. (1995). Neuraminidase is essential for fowl plague virus haemagglutinin to show haemagglutinating activity. *Virology* **212**, 77–83.

Ohuchi, M., Ohuchi, R., Feldmann, A., and Klenk, H.-D. (1997). Regulation of receptor binding affinity of influenza virus haemagglutinin by its carbohydrate moiety. *J. Virol.* **71**, 8377–8384.

Palese, P., and Compans, R. W. (1976). Inhibition of influenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid (FANA): mechanism of action. *J. Gen. Virol.* **33,** 159–163.

Palese, P., Schulmann, J. L., Bodo, G., and Meindl, P. (1974). Inhibition of influenza and parainfluenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid (FANA). *Virology* **59,** 490–498.

Rogers, G. N., Pritchett, T. J., Lane, J. L., and Paulson, J. C. (1983). Differential sensitivity of human, avian and equine avian influenza viruses to a glycoprotein inhibitor of infection: Selection of receptor specific variants. *Virology* **131,** 394–408.

Ryan, D. M., Ticehurst, J., and Dempsey, M. H. (1995). GG167 (4-Guanidino-2,4-dideoxy-2,3-dehydro-N-acetylneuraminic acid) is a potent inhibitor of influenza virus in ferrets. *Antimicrob. Agents Chemother.* **39**, 2583–2584.

Sauter, N. K., Glick, G. D., Crowther, R. L., Park, S.-J., Eisen, M. B., Skehel, J. J., Knowles, J. R., and Wiley, D. C. (1992). Crystallographic detection of a second ligand binding site in influenza virus haemagglutinin. *Proc. Natl. Acad. Sci. USA* **89,** 324–328.

Staschke, K. A., Colacino, J. M., Baxter, A. J., Air, G. M., Bansal, A., Hornback, W. J., Munroe, J. E., and Laver, W. G. (1995). Molecular basis for the resistance of influenza viruses to 4-guanidino-Neu5Ac2en. *Virology* **214,** 642–646.

Subbarao, K., Klimov, A., Katz, J., Regnery, H., Lim, W., Hall, H., Perdue, M., Swayne, D., Bender, C., Huang, J., Hemphill, M., Rowe, T., Shaw, M., Xu, X., Fukuda, K., and Cox, N. (1998). Characterisation of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science* **279,** 393–396.

Thomas, G. P., Forsyth, M., Penn, C. R., and McCauley, J. W. (1994). Inhibition of the growth of influenza viruses *in vitro* by 4-guanidino-2,4-dideoxy-N-acetylneuraminic acid. *Antiviral Res.* **24,** 351–356.

Thompson, W. R. (1947). Use of moving averages and interpolation to estimate median-effective dose. *Bacteriol. Rev.* **11,** 115–145.

van Deusen, R. A., Hinshaw, V. S., Senne, D. A., and Pellacani, D. (1983). Microneuraminidase-inhibition assay for classification of influenza virus neuraminidases. *Avian Dis.* **27**, 745–750.

von Itzstein, M., Wu, W.-Y., Kok, G. B., Peg, M. S., Dyason, J. C., Jin B., van Phan, T., Smythe, M. L., White, H. F., Oliver, S. W., Colman, P. M., Varghese, J. N., Ryan, D. M. M., Woods, J. M., Bethell, R. C., Hotham V. J., Cameron, J. M., and Penn, C. R. (1993). Rational design of potent sialidase-based inhibitors of influenza virus replication. *Nature* **363,** 418–423.

Weis, W., Brown, J. H., Cusack, S., Paulson, J. C., Skehel, J. J., and Wiley, D. C. (1988). Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. *Nature* **333,** 426–431.

Wilcoxon, F. (1945). Individual comparisons by ranking methods. *Biomet. Bull.* **1,** 80–83.

Woods, J. M., Bethell, R. C., Coates, J. A. V., Healy, N., Hiscox, S. A., Pearson, B. A., Ryan, D. M., Ticehurst, J., Tilling, J., Walcott, S. A., and Penn, C. R. (1993). 4-Guanidino-2,4-dideoxy-2,3-dehydro-N-acetylneuraminic acid is a highly effective inhibitor both of the sialidase (neuraminidase) and of growth of a wide range of influenza A and B viruses *in vitro*. *Antimicrob. Agents Chemother.* **37,** 1473–1479.

Yang, P., Bansal, A., Liu, C., and Air, G. M. (1997). Haemagglutinin specificity and neuraminidase coding capacity of neuraminidase-deficient influenza viruses. *Virology* **229,** 155–165.